Apolipoprotein J/Clusterin Is Induced in Vascular Smooth Muscle Cells After Vascular Injury

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Background—Understanding the precise molecular mechanisms underlying the phenomenon of restenosis after PTCA may help us to develop a new strategy for the treatment of restenosis after PTCA. The purpose of this study was to identify the genes involved in vascular restenosis.

Methods and Results—Applying a differential hybridization method to a model of the balloon-injured rabbit aorta, we identified 6 cDNA clones that were upregulated after injury. Northern blot showed that 5 genes, but not apolipoprotein J (apoJ)/clusterin, were constitutively expressed in noninjured aorta and upregulated after balloon injury. ApoJ mRNA was not detectable in noninjured aorta (control), began to be expressed at 6 hours after injury, showed a peak level at 24 hours (a 48-fold increase), gradually declined, and returned to the control level at 24 weeks. Western blot and immunohistochemistry demonstrated no expression of apoJ protein in noninjured aorta, an expression of apoJ at 2 days after balloon injury, and a peak level (a 55-fold increase) at 2 to 8 weeks. The expression of apoJ protein continued until 24 weeks after injury. In situ hybridization revealed that apoJ mRNA was expressed in smooth muscle cells (SMCs) of media at 2 days after injury and in SMCs of media and neointima at 2 weeks. To analyze the function of apoJ, stably transfected rabbit SMCs were created. The expression of apoJ stimulated proliferation and migration of SMCs.

Conclusions—ApoJ is dramatically induced in media and neointima after vascular injury, suggesting that apoJ contributes to restenosis after angioplasty. (Circulation. 2001;104:1407-1412.)

Key Words: angioplasty n restenosis n muscle, smooth n apolipoproteins

Percutaneous transluminal coronary angioplasty (PTCA) is a useful technique for treating patients with coronary atherosclerosis. The long-term efficacy of PTCA, however, is limited by vascular restenosis, which occurs in up to 40% of patients undergoing this procedure.1 Restenosis after PTCA is a consequence of balloon-induced smooth muscle cell (SMC) migration, proliferation, matrix production, and remodeling. Numerous attempts to prevent restenosis after PTCA have met with very limited success. This failure reflects the complexity of the pathophysiological process of restenosis. Our understanding of the cellular and molecular mechanisms of restenosis has made it difficult to identify appropriate cellular or molecular targets for therapy of restenosis after PTCA.

Intimal thickening and constrictive remodeling are main elements of the process of restenosis, and SMCs play a key role in this phenomenon. Balloon catheter injury to the artery wall induces the migration and proliferation of and matrix production by arterial SMCs, resulting in neointima formation. This animal model is frequently used to study vascular restenosis.2 Using this in vivo model, we performed a differential hybridization method to compare gene expression before and after vascular injury. This type of hybridization method has been successfully used to identify many differentially expressed genes.

The mechanism of restenosis after balloon injury is believed to involve cascades of autocrine and paracrine cytokine and growth factor signaling after vascular injury.3 There may be a master gene that controls this cascade. To begin to search for such a gene, we used differential hybridization to identify mRNAs that showed increased levels of expression at 24 hours after balloon injury in a rabbit aorta model.

Methods

Rabbit Aorta Model Male Japanese white rabbits (2.5 kg, 6 months old) were used in all experiments. All surgical procedures on the animals were carried out under general anesthesia with sodium pentobarbital (40 mg/kg body wt), ketamine (2 mg/kg body wt), and xylazine (8 mg/kg body wt) IV. Injury of the aorta was performed according to a standard protocol4 with minor modifications. Briefly, a 4F Fogarty balloon catheter (Baxter Healthcare Co) was inserted into the femoral artery and advanced to the aortic arch. The balloon was then inflated and...
passed 3 times along the length of the aorta. The balloon catheter was removed, and the femoral artery was permanently ligated. At specified time points after injury, animals were anesthetized, killed, and perfused with PBS, and the aortas were harvested. For RNA isolation, the adventitia was stripped away from the aorta with fine forceps in PBS and then snap-frozen in liquid nitrogen. Sham-operated animals, anesthetized and operated on without catheter insertion, were used as controls. This study was carried out in accordance with the Guide for Animal Experimentation, Faculty of Medicine, Kagoshima University.

RNA Isolation
Total RNA for use in the differential hybridization and Northern blot analysis was isolated by the standard guanidinium thiocyanate method. For differential hybridization, poly (A)+ RNA was prepared with the polyATract mRNA Isolation System (Promega) according to the manufacturer’s instructions.

Differential Hybridization
By use of a cDNA library construction kit (Stratagene), a directional cDNA library was constructed in a AZAP II phage vector with poly (A)+ RNA extracted from the balloon-injured aorta. The cDNA library was plated, and 2 replica nitrocellulose filters were made from each plate. The duplicate filters were hybridized with 32P-labeled single-strand cDNA probes generated with poly (A)+ RNA from either the balloon-injured aorta or noninjured (control) aorta by use of avian myeloblastosis virus reverse transcriptase (Promega) and [32P]dCTP (3000 Ci/(mmol/L); NEN-Dupont). The phage plaques that preferentially hybridized to the probe derived from the balloon-injured aorta were picked and rescreened twice. Plasmids were generated from consistently positive plaques by an in vivo rescue procedure according to the manufacturer’s protocol (Stratagene). Plasmid DNA was isolated for sequence analysis and for the preparation of a radioactive DNA probe by a random priming reaction, which was used for the Northern blot analysis.

DNA Sequence and Analysis
Automated direct sequencing of the cDNA clones was performed in both directions with some primers and an Applied Biosystems 310 DNA sequencer with the ABI BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Sequences were compared by use of the BLAST alignment algorithm network service.

Northern Blot Analysis
Northern blot was performed as described previously. GAPDH probe (Clontech) was used as control, and all densitometric data of the Northern blot were normalized for GAPDH signals. For quantification, images were analyzed with NIH Image software.

Western Blot Analysis
For Western blot analysis, aortas were ground to a fine powder under liquid nitrogen and incubated in ice-cold 0.1% Triton lysis solution (mmol/L: HEPES 10 [pH 7.4], sodium pyrophosphate 50, NaF 50, EDTA 5, EGTA 5, and NaCl 50; and 100 μmol/L Na3VO4, 0.1% Triton X-100, 500 μmol/L PMSF, and 10 μg/mL leupeptin) for 30 minutes. Insoluble matter was removed by centrifugation, and the protein concentration was measured by bicinchoninic acid assay (Pierce). Equal amounts of protein (20 μg) were run on a gel. Western blot was performed as described previously with primary antibody against human apolipoprotein J (apoJ) (Rockland). It was repeated 4 times, and similar results were obtained. For quantification, images were analyzed with NIH Image software.

Immunohistochemical Staining
Immunohistochemical staining was performed as described previously by the labeled streptavidin-biotin complex method [Histofine SAB-PO(G) or (M) kit, Nichirei]. A goat polyclonal antibody against human apoJ (Rockland) or a mouse monoclonal antibody against muscle actin (Enzo Diagnostics) was used as primary antibody. The specificity of the immunoreaction was evaluated in comparison with a negative control specimen in which goat or mouse IgG was used instead of primary antibody.

In Situ Hybridization
In situ hybridization was carried out as reported previously with thymine-thymine (T-T) dimerized synthetic oligonucleotides complementary to a rabbit apoJ mRNA as probe. A 45-base sequence (italic type) complementary to the mRNA of rabbit apoJ (345–389) was chosen. A computer-assisted search (GenBank) of the antisense sequences, as well as the sense sequences, revealed no significant homology with any known sequences other than that of apoJ. For haptenization of the oligo-DNAs with T-T dimers, 2 and 3 TTA repeats of the 5′ and 3′ ends of the sequences were added, respectively, as follows: antisense probe, TTATTATTCCGTAGCAGTCGTGCCCCATTATT; sense probe, TTATTAAAGCCTCAGGCGATTCCGGAGATGGTGCACAGGACACCAGTGGCGATTATT.

Stably Transfected Cell Lines
Rabbit SMCs were isolated from the media of rabbit aortas. ApoJ cDNA was ligated into the mammalian expression plasmid vector pcDNA3.1 (Invitrogen). Rabbit SMCs, in 35-mm culture dishes, were transfected with 5 μg of the expression plasmid by use of lipofectamine (Life Technologies). The following day, the cells were trypsinized, plated into 100-mm culture dishes, and treated with 800 mg/mL Geneticin (Life Technologies) to select for cells stably integrating the neo gene. Individual colonies were picked and expanded to test for expression of the transfected gene by Northern blot.

SMC Proliferation and Migration Assay
SMC proliferation was determined by measurement of [3H]thymidine incorporation in SMCs as described previously. The chemoactive migration of SMCs was measured with a transwell migration apparatus. M199 medium with 10% FBS was added to the lower wells of the transwell chamber (Chemotaxicell, Kurabou). Transfected SMCs were trypsinized, resuspended in M199 with 0.5% BSA at a density of 5×104 cells/mL, and added into the upper wells of the transwell chamber with a PVP-free filter with 8-μm pores. The chambers were incubated for 6 hours, and cells were fixed and stained with hematoxylin. All migrated cells attached to the bottom of the filter were counted. All the experiments were performed in quadruplicate, and each experiment was repeated 4 times. An unpaired Student’s t test was used for statistical analysis.

Results
mRNA was isolated from control (noninjured) and balloon-injured rabbit aortas at 24 hours after injury, and differential hybridization was performed to identify changes in gene expression that occur in the SMCs of aorta after balloon injury. After tertiary screening, 6 overexpressed phage plaques were obtained. These 6 cDNA clones were sequenced and analyzed by searching for homologies against the GenBank database. Homologues to the following sequences were found: apoJ/clusterin, importin-β, tumor-associated protein, α-globin, α-actin, and mitochondrial DNA.

With these 6 cDNA clones used as probes, Northern blot analysis was performed to confirm the gene regulation patterns observed in the differential hybridization (Figure 1; data for α-actin and mitochondrial DNA are not shown). Although an equal amount of total RNA was loaded, as shown by the intensity of the 28S ribosomal RNA of each lane, the GAPDH mRNA level was increased 24 hours after vascular injury (a 1.5-fold increase). Therefore, the expres-
The expression level of genes was normalized for GAPDH. Northern blot analyses of control and balloon-injured aortas obtained 24 hours after balloon injury showed that all the identified genes, except for apoJ, were constitutively expressed in the noninjured aorta and upregulated at 24 hours after balloon injury. Of these, apoJ mRNA was the most significantly induced after injury (a 48-fold increase, Figure 1), as assessed by Northern blot analysis. Therefore, we focused on apoJ.

The cDNA of rabbit apoJ was sequenced in both directions. This cDNA contained the full-length cDNA of apoJ deduced from other species and had an open reading frame of 447 amino acid residues with a hydrophobic signal sequence (GenBank accession No. AF118852). By Northern blot analysis using this cDNA, the time course of mRNA expression of apoJ after balloon injury was analyzed (Figure 2). ApoJ mRNA was not detectable in noninjured aorta (control), began to be expressed at 6 hours after injury, showed a peak level at 24 hours (a 48-fold increase compared with control), gradually declined, and then returned to the control level at 24 weeks after injury.

To quantify the expression level of apoJ protein, Western blot analysis was performed with primary antibody against human apoJ (Figure 3). Densitometric analysis of 4 independent experiments demonstrated no expression of apoJ protein in noninjured aorta (control), began to be expressed at 6 hours after injury, showed a peak level at 24 hours (a 48-fold increase compared with control), gradually declined, and then returned to the control level at 24 weeks after injury.

To localize the expression of apoJ protein in the balloon-injured rabbit aorta, we performed immunohistochemical staining with polyclonal antibodies raised against human apoJ (Figure 4). ApoJ protein was not expressed in the media of noninjured aortas. It was expressed, however, in the SMCs of the media at 2 days after injury and in the SMCs of the media and neointima at 2 and 8 weeks after injury (Figure 4) and 24 weeks after injury (data not shown). To confirm that apoJ exists in SMCs, immunohistochemical staining for muscle actin was also performed on serial sections from aorta 2 weeks after injury. It showed that not only media but also neointima was composed predominantly of SMCs (Figure 4f).

To confirm that the SMCs were synthesizing apoJ, in situ hybridization analysis was performed (Figure 5). No apoJ mRNA was detected in the media of control aortas, whereas mRNA for apoJ was expressed in the SMCs of the media at 2 days after injury and the SMCs of the media and neointima at 2 weeks after injury.

To examine the effects of apoJ on proliferation and migration of SMCs, stably transfected rabbit SMC cell lines were established. Northern blot demonstrated robust expression of apoJ mRNA in apoJ-transfected cells (data not shown). On 10% FBS or 10 ng/mL platelet-derived growth factor medium, [3H]thymidine incorporation in apoJ-transfected SMCs was significantly higher than that in SMCs transfected with plasmid vector (control) (Figure 6A).
number of migrating apoJ-transfected SMCs induced by 10% FBS was significantly higher than that of control (Figure 6B).

**Discussion**

Although many reports have focused on gene expression in proliferating and migrating SMCs after vascular injury, this is the first report to use differential hybridization to focus on an early time point, 24 hours after balloon injury. We chose this early time course to identify master genes controlling the cascade reaction leading to neointimal formation after vascular injury. The differential hybridization method is a powerful technique for identifying differentially expressed genes. In this study, 6 balloon-injury–induced genes were identified, including apoJ/clusterin, importin β, tumor-associated protein, α-globin, α-actin, and mitochondrial DNA.

Because apoJ mRNA is dramatically induced after balloon injury, we focused on apoJ in this article. ApoJ is a highly conserved secretory glycoprotein and is identical to the proteins previously named clusterin, serum protein 40-40, testosterone-repressed protein message-2, sulfated glycoprotein-2, and complement lysis inhibitor. ApoJ has been found in most physiological fluids, including human plasma, urine, breast milk, semen, and cerebrospinal fluid.13,14 The wide distribution and sequence conservation of apoJ suggests that this protein performs functions of fundamental biological importance. These include lipid transport,15 sperm maturation,16 regulation of the complement cascade,17 apoptosis,18 and membrane recycling.19

Using immunohistochemistry and in situ hybridization, we confirmed that the protein and mRNA for apoJ exist in SMCs of the media and neointima after balloon injury. Although the mRNA of apoJ vanished at 24 weeks after injury by Northern blot analysis, a high level of apoJ protein was maintained at 24 weeks by Western blot and immunohistochemistry. The reason for this discrepancy is thought to be that apoJ accumulated in the media and neointima of aorta at 24 weeks, because apoJ is distributed in the intima and media of human aortas with intimal thickening or atherosclerotic lesion.20

Several investigators have reported that apoJ is induced after tissue injury or inflammation in a variety of tissues. Swerdlow et al21 reported that apoJ protein accumulates in myocytes at the interface between degenerated myocardial tissue and the surrounding cardiac tissue in myosin-induced and viral myocarditis. These authors proposed that apoJ functions to limit and/or promote tissue injury. In another report, the apoJ mRNA was found to be upregulated in kidney epithelial cells after obstruction by ureteral ligation compared with the contralateral nonobstructed kidney.22 Therefore, apoJ is likely to be involved in the process of tissue remodeling.

Cordero et al23 reported that the expression of a clone with strong homology to apoJ was significantly increased in a distal anastomotic artery segment compared with normal
artery segments in the dog. The anastomotic intimal hyperplasia is similar to that seen after balloon injury. Both of these are considered to be forms of accelerated atherosclerosis. In addition, we confirmed that the apoJ protein was expressed in human atherectomy specimens from restenotic lesions after PTCA and transluminal femoral angioplasty using immunohistochemistry (unpublished data). To analyze the function of apoJ, stably transfected rabbit SMC cell lines were created. The expression of apoJ stimulated proliferation and migration of vascular SMCs in vitro, suggesting that apoJ contributes to restenosis after angioplasty. Further in vivo studies are needed to determine the role of apoJ in restenosis after PTCA.

Importin is the nuclear import receptor for nuclear localization signal (NLS)–containing proteins. NLS proteins are transported into the nucleus by the importin-α/β heterodimer. Importin-α binds NLS, whereas importin-β mediates translocation through the nuclear pore complex. Several observations suggest that nuclear transport plays a key role in proliferation of cells through mitosis. Therefore, importin-β may play a role in SMC proliferation after balloon injury.

The gene encoding tumor-associated protein was isolated from a cDNA library made from the midlactating (14 days) rabbit mammary gland and corresponds to casein. There is a possibility that this gene may be associated with transcription of some proteins during SMC proliferation. Further examination is necessary, however, to clarify the function of this gene in neointimal formation after balloon injury.

The function of α-globin in neointimal formation after balloon injury is not known and remains to be determined. De Leon et al. reported that α-globin expression decreases after sciatic nerve injury. Thus, α-globin may play a role in tissue remodeling after injury in both nerve tissue and artery.

Both mitochondrial DNA and α-actin are necessary for the proliferation of SMCs. Given that SMC proliferation is a hallmark of neointimal formation after vascular injury, upregulation of mRNA for mitochondrial DNA and α-actin after balloon injury is not surprising.

In conclusion, balloon injury–induced genes were isolated by a differential hybridization technique to identify genes involved in restenosis after PTCA. ApoJ is dramatically induced in media and neointima after balloon injury, and stably transfected rabbit SMCs showed that the expression of apoJ stimulated proliferation and migration of SMCs. These results suggest that apoJ contributes to restenosis after angioplasty.
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